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Apoptosis of rabbit retinal cell after eyeball rupture

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ABSTRACT

Objective: To establish rabbit eyeball rupture model by air gun in order to observe and analyze the early injury condition and reasons of retinal cell after eyeball rupture. **Methods:** Forty eight healthy rabbits were randomly divided into control group and 1, 3, 6, 12 and 24 h after injury groups. After anesthesia, the rabbit eyeball rupture model was established by air gun. Then the early pathological changes of rabbit retina were observed, and apoptotic index (AI), oncosis index (OI), the relationship between the expression amounts of apoptosis-related genes and AI were analyzed. **Results:** Obvious pathological lesion appeared in retina 6 h after injury. Irreversible damage occurred 12-24 h after injury. The results of AI and OI indicated that the OI peak appeared 6 h after injury and then gradually declined, while the AI increased with the prolongation of time, and the AI was higher than OI in 12 h after injury. Immunohistochemical results indicated that there was no obvious bcl-2 protein expression change. Compared with the control group and the 3, 6, 12 and 24 h after the injury groups, the expressions of p53 and Caspase-3 were significantly improved and peaked at 12 h ($P<0.01$). Positive correlation existed among p53, Caspase-3 expression amount and cell apoptosis amount. **Conclusions:** Apoptosis and oncosis of visual cells are the main reasons of retinal cell injury. p53 and Caspase-3 are the important factors in promoting the retinal cell apoptosis after eyeball rupture.

1. Introduction

The incidence rate of eye injury is 2%-10% in war. The traumatogenic factor of most eye injury in war is weapon or fragment of explosion. Eye rupture and penetrating injury are the most common in eye injuries in war. As a kind of severe open ocular trauma, eyeball rupture often combined with intraocular content loss and severe posterior pole retinal damage is called severe open ocular trauma characterized as poor prognosis and high blindness rate. The current therapeutic means cannot effectively improve cell death, cicatrization, and hyperblastosis and ganglion cell axon hyperplasia, *etc*[1]. Lots of studies verified that apoptosis participates in the ischemia-reperfusion, light injury, retinal seperation and the photoreceptor cell injury of ocular contusion[2-4]. Ophthalmological studies mainly focus on hypertrophic vitreoretinopathy after injury[5-7], retinal response after injury (including retinal ganglion cell apoptosis[8-11], ganglion cell axon hyperplasia[12-14] and inflammatory reaction)[15,16], and retinal cell death and remodeling, *etc*[17,18].

p53, bcl-2 and Caspase-3 are important genes related to cell apoptosis. As an anti-oncogene, p53 has the function of transcription factor with its encoding protein in the nucleus. When cell is injured, wild-type p53 protein will timely initiate apoptosis in order to maintain the stability of the internal environment[18,19]. As an oncogene[20], with its encoding protein in the surface of mitochondrial inner membrane in cytolymph, bcl-2 can block the transmission of apoptotic signal, inhibit apoptosis and prolong the cell life. Caspase-3 is a known key molecule in the process of apoptosis, playing an important role in the execution of apoptosis. Some studies have shown that Caspase-3 activation is closely related to the apoptosis of pigment epithelial cell, outer nuclear layer cell and ganglion cell

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caused by retinal injury[21-23]. In this study, eyeball rupture model was built. Then we observed the early clinical manifestation and tissue histopathological change of retina after rabbit eyeball rupture, the expression of p53, bcl-2 protein and Caspase-3 in retinal cells, the cell apoptosis of retinal cells and the spatial and temporal distribution of the swelling and necrosis retinal cells in order to provide experimental basis for the clinic treatment.

2. Materials and methods

2.1. Animal, reagents and instruments

After anesthesia, 48 healthy male New Zealand white rabbits, without eye diseases (turn out normal after the examination of outer eye and fundus), provided by the Experimental Animal Center in Henan Province, were selected in this experiment. Then they were randomly divided into control group and 1, 3, 6, 12 and 24 h after injury groups (experimental group), six groups in total and eight rabbits in each group. 0.4% eye drops (Santen Pharmaceutical Co., Ltd., Japan) and 3% pentobarbital sodium (Sigma Co., Ltd., USA) were prepared. Cornea confocal microscopy was from Japan NIDEK Company. Air gun was purchased from Chongqing Hongyuan Machinary Co., Ltd.

2.2. Establishment of the eyeball rupture model

This project was approved by the ethics committee of Zhengzhou University. Animal experiments conform to institutional standards. The care and use of animals was in accordance with institutional and national guidelines and in accordance with legal requirements in China. We adhered to the tenets of the Declaration of Helsinki or the NIH statement for the use of Animals in Research. All procedures were approved by the Institutional Animal Care and Use Committee and were carried out according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The rabbits were anesthetized by intravenous injection of pentobarbital sodium from ear limbus (dose: 30 mg/kg×weight), and according to the degree of recovery, 1/5-1/4 of the initial dose was injected in order to sustain the anaesthesia[24]. After anaesthesia, the rabbit's eyes were shot from the rabbit's ear root along the line between ear root and center of the eyeball by an air gun, with the gunpoint 1 mm away the corneoscleral limbus, and the bullet was shot out of the nose root forming the anterior part eyeball rupture model. Then debridement and suturing was performed, and tobramycin was injected under conjunctiva. Penicilin was intramuscularly injected. 1, 3, 6, 12 and 24 h after injury, the rabbits was euthanized. Then the eyeball was immediately removed to detect the retinal morphological and functional changes.

2.3. Retinal electrophysiological observation

Electroretinogram (ERG) test was performed 1 h, 3 h, 6 h, 12 h and 24 h, respectively after injury[25]. Detection method: after full mydriasis and 30 min dark adaptation, SDY visual electrophysiological instrument was used to detect the change of ERG-b wave. The amplitude and peak value of ERG b wave were observed and detected.

2.4. p53, bcl-2 and Caspase-3 expression detected by immunohistochemical method and retinal morphological change

After enucleation of eyeball in the state of anesthesia at different time points, the surface blood in extracted eyeballs was washed by physiological saline. After cornea resection, the eysballs were fixed for 24 h by 4% paraformaldehyde phosphate buffer. Then dehydration by alcoholic in gradient concentration, xylene transparent solution and paraffin embedding were performed. Four serial sections of posterior polar retina were resected with the 4 μm slice thickness. HE staining and MG-P-MY staining was respectively given to each two of them. Then the sections were put on the APES pretreated glass slide and put in the incubator overnight at 60 ℃. The steps of immunohistochemistry: (1) The dehydrated paraffin section was put into the 3% H_2O_2 solution for 10 min at room temperature, and then washed three times by distilled water; (2) The slide was soaked in the citric acid solution and heated to boiling by microwave oven. After 5 min-interval, this process was repeated twice; (3) The BSA confining liquid was added and set aside for 20 min at room temperature, and then the redundant solution was removed; (4) The rabbit anti-human antibody (or p53 antibody or Caspase-3 antibody) was added and overnight at 4 ℃. The sections were washed three times with 2 min for each time; (5) The biotinylated goat anti-rabbit IgG was added. The section was incubated in incubator for 20 min at 37 ℃. Then, the section was washed by PBS (pH=7.4) for three times with 5 min at a time; (6) SABC reagent was added. The section was incubated in incubator for 20 min at 37 ℃. Then, the section was washed by PBS (pH=7.4) for three times with 5 min at a time; (7) DAB coloration; (8) Slight restaining by hematoxylin, dehydration, transparence and mounting were conducted for microscope observation.

The distribution of positive RNA expression and apoptosis of retinal cells were detected by methyl green-pyronine-Matthew Huang (MG-P-MY) staining method[26,27].

Positive RNA cells (apoptotic cells) were pink or dark red stained under the observation of light microscope.

The following three kinds of cells can be seen under the ultramicroscope, normal cell with green dyed nuclear, pink apoptotic cell and swelling necrotic cells with reticular green dyed chromatin. The apoptotic index (AI) (AI= Number of positive RNA cells/total number of cells ×100%), number of oncosis cells and oncosis index (OI) (OI= Number of oncosis cells/total number of cells ×100%) were respectively calculated.

2.5. Statistical processing and data linear regression analysis

Statistical processing was performed by SPSS10.0 software. Firstly, test of homoscedasticity and normality was conducted. If heterogeneity of variance existed, change of variable was performed. After homogeneity of variance, the data difference of different time points in the same group was analyzed by Oneway-ANOVA. The least significant difference method was adopted to make the pairwise comparison between means. The relationship between two variables was analyzed by Spearman rank correlation analysis. Size of test: α =0.05. Regression analysis was performed for the correlation of data in each group by regress function in Matlab 7.0 software.

3. Results

3.1. ERG detection results

The ERG-b wave average amplitude of the eight rabbits in the control group was (103.50±16.84) mv, while the latent time was (97.30±6.58) ms. The ERG-b waves in the 1 h, 3 h, 6 h, 12 h and 24 h after injury group were evanescent waves (Figure 1).

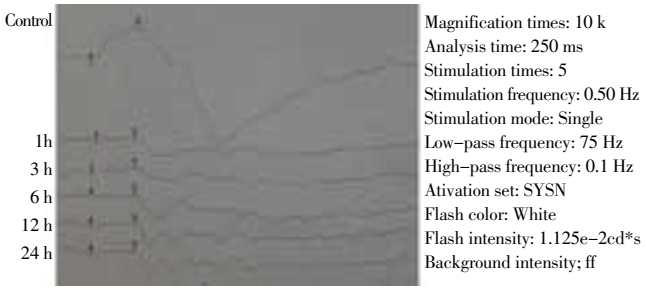

Figure 1. Electroretinogram.

3.2. Pathological test

Ordinary light microscope showed clear normal retinal ten-layer structure. 1 h after injury, fracture occurred in the retinal cone-rod cells, and there was no obvious change in the inner and outer nuclear layer and ganglion cells. There was slight hematocele in the surface of retina; 3 h after injury, there was comparatively obvious fracture of cone-rod cells in the retinal cone-rod cells. Endochylema loose in inner nuclear layer and several vacuoles degeneration occurred. There was no obvious change in outer nuclear layer. The nuclear of ganglion cells was slightly stained. Obvious edema of nerve fiber layer can be observed. 6 h after injury, the fracture of cone-rod cells was obvious. Obvious vacuoles degeneration occurred in ganglion cells and inner nucleus layer. The number of ganglion cells began to decrease, and the layers of outer nuclear layer cell decreased. 12 h after injury, the number of ganglion cells and inner nuclear layer cells significantly decreased. The outer nuclear layer cells were arranged in disorder, and karyopyknosis can be observed. 24 h after injury, the retinal structure was in disorder. The cone-rod layer was fractured and disappeared. Obvious vacuoles degeneration occurred in the inner and outer nucleus layer, and the number of which was significantly decreased. The karyolysis and pyknosis of the nuclear of ganglion cells continued (Figure 2).

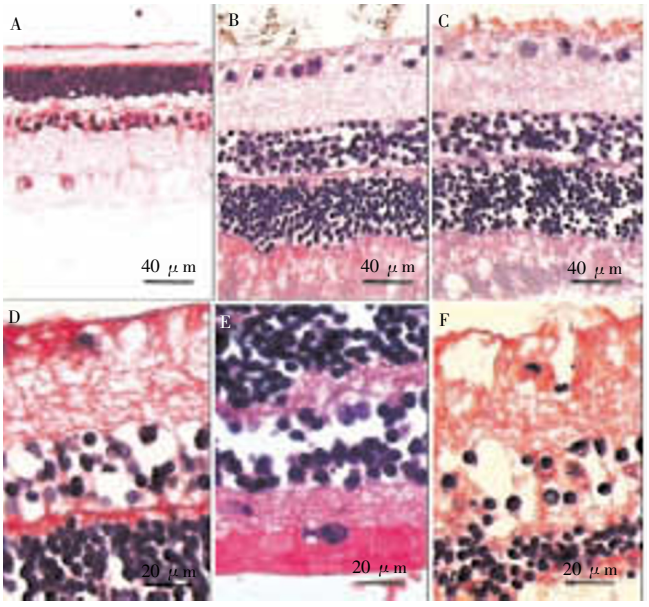

Figure 2. HE staining of retina after injury.
A: normal retina ×200; B: retina of 1 h after injury ×200; C: retina of 3 h after injury ×200; D: retina of 6 h after injury ×400; E: retina of 12 h after injury ×400; F: retina of 24 h after injury ×400.

3.3. Results of MG-P-MY staining

No positive RNA expression (no apoptotic cell) was detected in retinal tissue of the 1 h group. There was few positive RNA expressions in the inner nucleus layer and ganglion cell layer in the 3 h group. In the 6 h group, the positive cells in inner nucleus layer increased, and few positive RNA expressions in outer nucleus layer appeared. In the 12 h group, obviously positive cells appeared. Large amount of RNA positive cells can be seen in the 24 h group (Figure 3). Compared the AI of control group with each experimental group, there was significant difference between the control group and the 1 h after injury group (P=0.033). However,

there was no significant difference between the control group and the other experimental groups (P<0.01) (Table 1).

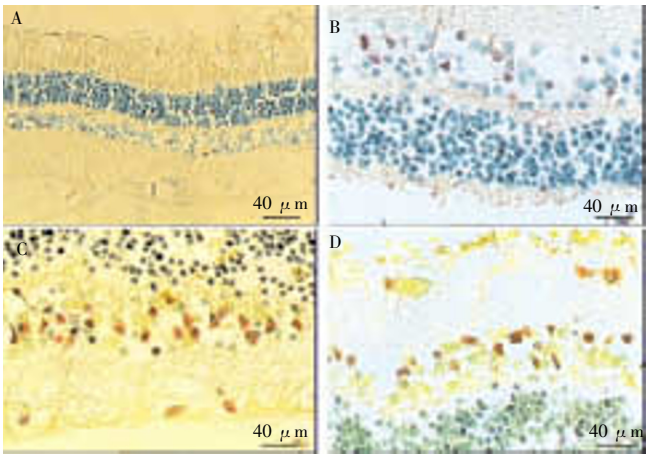

Figure 3. RNA positive cells in retinal tissue in each time points detected by MG-P-MY staining (×200).
A: 3 h after injury; B: 6 h after injury; C: 12 h after injury; D: 24 h after injury.

The observation by ultramicroscope: No necrosis cells can be seen in the 1 h group. Necrosis cells appeared in 3 h reached the peak at 6 h and slightly decreased from 12 h.

Table 1
AI and OI of retinal cell in the control group and each experimental groups($\bar{x}$±s).

Group	AI	OI
Control	0.003 5±0.002 9	0.007 3±0.003 8
1 h after injury	0.035 9±0.075 4	0.067 4±0.003 9
3 h after injury	0.057 2±0.035 7	0.187 1±0.009 2
6 h after injury	0.187 6±0.066 0	0.338 4±0.053 8
12 h after injury	0.327 9±0.054 9	0.284 9±0.664 3
24 h after injury	0.358 0±0.087 0	0.218 6±0.077 9

Comparison of apoptotic cell (RNA positive cell) with swelling and necrotic cell: No necrosis cells can be seen in the control group and 1 h group. Necrosis cells appeared in 3 h, reached the peak in 6 h and slightly decreased in 12 h. From the aspect of cell percentage, within 6 h (including 6 h) after injury, the percentage of oncosis cells was larger than that of the apoptotic cells. 12 h (including 12 h) after injury, the percentage of apoptotic cells was larger than that of the oncosis cells. With prolongation of time, the number of oncosis and swelling cells showed the tendency of decline. However, the number of apoptotic cells tended to stabilization although slight augmentation can be seen 12 h after injury.

3.4. Expression of bcl-2, p53 and Caspase-3

bcl-2 protein was relatively low expression in the control and each experimental group, and there was no significant difference after homogeneity test for variance and analysis of variance after single factor multiple comparisons (P>0.05). Brownish yellow nucleus indicated that p53 was positively expressed. In this experiment, no brownish yellow staining can be seen in the normal retinal tissue, that is, p53 was not expressed. One hour after injury, seldom p53 was expressed in retina. 3 h after the injury, few p53 expressions can be found. p53 positive expression was obvious 6 h after injury and peaked in the 12 h. p53 mainly expressed in the inner nucleus layers and ganglion cell layer, and few expressed in the outer nucleus layer cell (Figure 4). bcl-2 and p53 protein expression and their correlation with cell apoptosis in the control group and each experimental group were demonstrated in Table 2, 3, 4. After homogeneity test for variance and single factor multiple comparison, there was significant difference between the control group and 3 h, 6 h, 12 h and 24 h group after the injury (P<0.01). Regression analysis of the relationship between p53 protein expression and cell apoptosis was performed by the regress function in Matlab 7.0 software (using p53 protein expression as the independent variable x and retinal cell apoptosis as the dependent variable y). The equation of linear regression: y=0.926 6x+1.572 3 (Figure 5).

Table 2
Average optical density value of bcl-2, p53 and Caspase-3 expression amount in retinal tissue ($\bar{x}$±s).

Group	bcl-2	p53	Caspase-3
Control	2.19±0.37	0.81±0.40	2.43±1.06
1 h after injury	1.94±0.27	1.78±0.86	5.75±1.28
3 h after injury	2.56±0.38	9.19±1.83	8.48±2.91
6 h after injury	2.37±0.25	8.06±4.72	17.64±3.57
12 h after injury	2.00±0.35	32.88±5.98	27.38±4.37
24 h after injury	2.53±0.35	17.50±3.43	22.86±2.56

Table 3
Correlation analysis between bcl-2 and apoptosis.

Group	bcl-2($\bar{x}$±s)	Apoptosis($\bar{x}$±s)	Correlation coefficient (r)	P
Control	2.19±0.37	0.44±0.32	-0.109	0.797
1 h after injury	1.94±0.27	1.50±0.52	-0.338	0.412
3 h after injury	2.56±0.38	3.06±1.15	-0.679	0.064
6 h after injury	2.37±0.25	15.25±5.50	0.135	0.749
12 h after injury	2.00±0.35	28.25±7.21	-0.404	0.321
24 h after injury	2.53±0.35	26.00±6.93	0.339	0.412

Table 4
Correlation analysis between p53 and apoptosis.

Group	p53 ($\bar{x}$±s)	Apoptosis($\bar{x}$±s)	Correlation coefficient (r)	P
Control	0.81±0.40	0.44±0.32	0.884	0.004
1 h after injury	1.78±0.86	1.50±0.52	0.902	0.002
3 h after injury	9.19±1.83	3.06±1.15	0.741	0.035
6 h after injury	8.06±4.72	15.25±5.50	0.827	0.011
12 h after injury	32.88±5.98	28.25±7.21	0.965	0.000
24 h after injury	17.50±3.43	26.00±6.93	0.847	0.008

Table 5
Correlation analysis between Caspase-3 and apoptosis.

Group	Caspase-3 ($\bar{x}$±s)	Apoptosis($\bar{x}$±s)	Correlation coefficient (r)	P
Control	2.43±1.06	0.44±0.32	0.903	0.003
1 h after injury	5.75±1.28	1.50±0.52	0.894	0.005
3 h after injury	8.48±2.91	3.06±1.15	0.857	0.016
6 h after injury	17.64±3.57	15.25±5.50	0.925	0.009
12 h after injury	27.38±4.37	28.25±7.21	0.971	0.001
24 h after injury	22.86±2.56	26.00±6.93	0.887	0.004

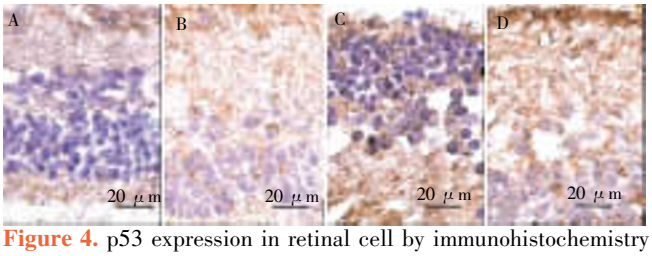

Figure 4. p53 expression in retinal cell by immunohistochemistry staining (×400).
A: p53 expression in control group; B: p53 expression 6 h after injury group; C: p53 expression 12 h after injury group; D: p53 expression 24 h after injury group.

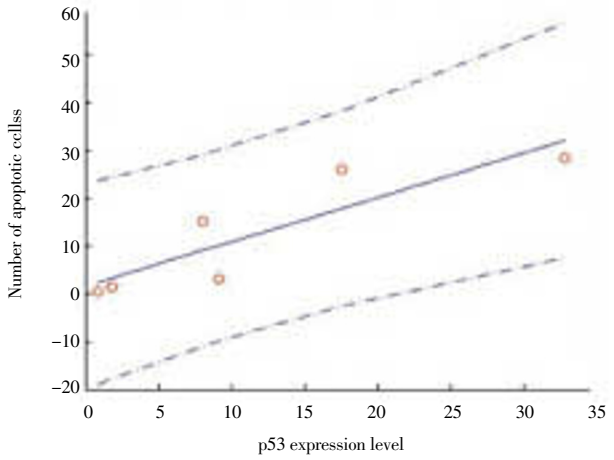

Figure 5. Linear relationship between the p53 expression and cell apoptosis in visual cells after injury.

One hour after injury, the Caspase-3 expression in the ganglion cell layer and inner nucleus layers began to significantly increase, and continued this trend 3 h and 6 h after the injury, finally peaked at 12 h, but declined from 24 h. Compared with the control group, there was significant difference (P<0.01) (Table 2, Table 5, Figure 6). Caspase-3 positive cell mainly distributed in the inner nucleus layer and ganglion cell layer and few distributed in the outer nucleus layer cell. Regression analysis of the relationship between Caspase-3 expression and cell apoptosis was performed by the regress function in Matlab 7.0 software (using Caspase-3 expression as the independent variable x and retinal cell apoptosis as the dependent variable y). The equation of linear regression: y=1.237 9 x−5.024 7 (Figure 7).

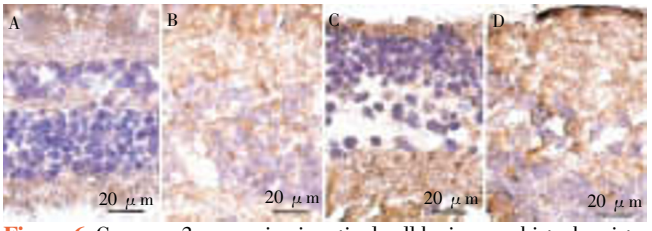

Figure 6. Caspase-3 expression in retinal cell by immunohistochemistry staining (×400).
A: Caspase-3 expression in control group; B: Caspase-3 expression 6 h after injury group; C: Caspase-3 expression 12 h after injury group; D: Caspase-3 expression 24 h after injury group.

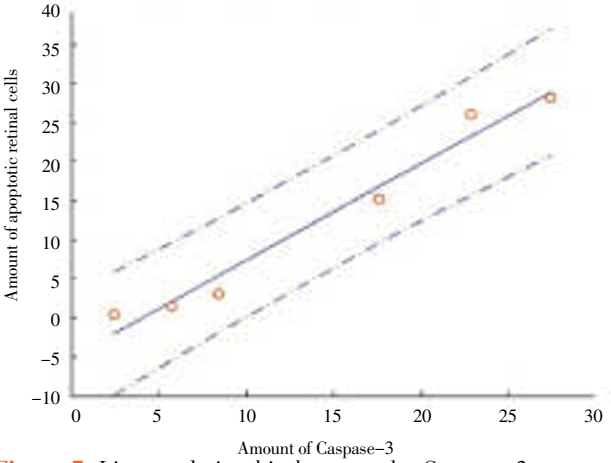

Figure 7. Linear relationship between the Caspase-3 expression amount and cell apoptosis in visual cells after injury.

4. Discussion

Compared with the control group in this experiment, there

was no obvious change in the expression of bcl-2, and bcl-2 was relatively low expression both in the control group and experimental groups after injury. It seemed that bcl-2 protein cannot inhibit cell apoptosis. The reason for this may be that bcl-2 may participate in the retinal cell apoptosis after severe open ocular injury[28,29]. In terms of time, 12 h after injury, the peak of p53 and Caspase-3 expression appeared before the retinal cell apoptosis peak, existing a time difference in regulation, which implied that the p53 and Caspase-3 overexpression after rupture may lead to the cell apoptosis. Caspase-3 may play a key role in the early stage apoptosis of retinal cell. This experiment verified that apoptosis existed in retinal photoreceptor cells in the early stage of rabbit severe open ocular trauma, and early anti-apoptotic treatment may help to protect the residual retina and benefit the severe open ocular trauma.

Previous literatures verified that retinal cell apoptosis took place in the eye injury like ischemia-reperfusion, retinal detachment, and ocular blunt trauma, *etc*[30-32]. In our study, the main methods of retinal cell death after eyeball rupture were apoptosis and oncosis. From the aspect of cell percentage, within 6 h (including 6 h) after injury, the percentage of oncosis cells was larger than that of the apoptotic cells. 12 h (including 12 h) after injury, the percentage of apoptotic cells was larger than that of the oncosis cells. With prolongation of time, the number of oncosis cells showed the tendency of decline. However, the number of apoptotic cells tended to stabilization although slight augmentation can be seen 12 h after injury. Therefore, apoptosis and oncosis were the main ways of retinal cell death in this model, and especially apoptosis of retinal cell contributed more to retinal injury, which also indicated the researchers that the apoptosis of ganglion cells and inner nuclear layer cells may influence the prognosis and visual recovery in a great degree.

Eyeball rupture is frequently occurred in soldiers. In normal times or in times of war, the incidence of eyeball rupture (or other ocular trauma) ranked the first in the hospitalized officers and soldiers in the Ophthalmology Department, and eyeball rupture is also the main reason causing visual loss and influencing battle effectiveness. In this experiment, the shooting direction and distance were fixed, and the shooting direction was from back to front when building the eyeball rupture model. In this way, the possible injury to other organs and tissues was avoided. Compared with previous eyeball rupture models[33,34], this model is the most similar to the eyeball rupture in war wounds. This research will help to provide new idea and strategy to the early and timely treatment of eye injury in war or emergent events.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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